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Characterization of Nrk2b Overexpression in a Transgenic Zebrafish Congenital Muscular Dystrophy Model

Anna Burgess
University of Maine - Main

CHARACTERIZATION OF NRK2BOVEREXPRESSION IN A TRANSGENIC ZEBRAFISH CONGENITAL MUSCULAR DYSTROPHY MODEL

by

Anna Burgess

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Advisory Committee:
Clarissa A. Henry, Associate Professor of Biological Sciences, Advisor
Harold Dowse, Associate Director of Biology and Ecology
Jim Gallagher, Associate Professor of Honors (Sociology)
Gregory Howard, Assistant Professor of English
Mary Tyler, Professor of Zoology

Abstract

Everyday movement, whether sitting or running a marathon, exerts stressful forces on myotendinous junctions (MTJs). As the sites which skeletal muscle fibers anchor to surrounding extracellular matrix (ECM) material of tendon, MTJsneed to maintain homeostasis under stress for muscle to function normally. Human congenital muscular dystrophies (CMDs) are a heterogeneous group ofdiseases that disrupt muscle homeostasis, are severely debilitating, and currently have no cure. In many CMDs, genetic mutationseffect cell adhesion complexes located at the MTJ. The adherence of muscle fibers' cytoskeleton to the extracellular matrix (ECM) is weakened when these complexes as compromised. Many genes resulting in muscular dystrophies are currently identified, yet the fundamental cell processes underlying muscle formation and attachment are chiefly unknown. This gap in knowledge results in a lack oftissue specific treatments. This project aims to research whether the overexpression of nicotinamide ribose kinase 2b (Nrk2b) enzyme, required in a salvage pathway to generate NAD+ and aid in the regulation of muscle cell adhesion, can be exploited to rescue a form of congenital muscular dystrophy in the zebrafish model organism. Exploring this pathway will potentially lead to knowledge that can be applied to gene therapies, using the Nrk2b kinase as a pharmacological target, and deepening knowledge of the zebrafish model system for the study of muscle disease.

Dedication

A great deal of what worked up to and what work went into my thesis is an unsettled debt to my grandmother. She continually anticipated more from me in a way that didn't demand or require. Resultantly, I hope I have learned to be demanding of myself in an anticipation of more. This piece of my learning I want to return to her. It is only a small bit for everything she gave to me (and all the times she sharply scoured the newspapers' academic sections for my name- daring me to not be there with a deliberate grin).

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Table of Contents

Introduction

Muscle Function: the journey of contraction

The primary function of muscle tissue is contraction. Whether taking notes in class or summiting a mountain, we depend on muscle contraction. Muscle fibers comprise highly regulated functional units known as sarcomeres. The primary machineries of sarcomeres are thin actin and thick myosin II filaments spatially and functionally regulated by proteins (Ross, 2011). Conciseness of design makes sarcomeres strong, efficient workers capable of generating power-strokes that cause contraction.

Contractile forces are fundamental to voluntary movement and stable posture. Contraction is the *means* eventually translated into an *end* at the skeleton. An important question of muscle formation and function is what comes in between. How is contraction orchestrated into function? The answer is not straightforward or completely understood. An aim of this study is to elucidate the complexities bridging muscle contraction and function. Pursuing knowledge of pathways and structures instrumental in normal function is desirable. It is a vital source for biomedical research, for the knowledge can be exploited to develop new therapies for muscle disease and dysfunction.

To illustrate the complexities of translating contraction to function, contractile forces can be approximated to an automobile on a road trip. A road is essential for the so-called contracto-mobile to reach a skeletal destination. To facilitate a successful journey, the road is constructed with infrastructure and equipped with signaling lights. Being such a common activity, it is easy to overlook possible dangers of travel. High speeds and faulty wiring are potential hazards resulting in collision and breakdown. The consequences compromise the contrato-mobile's safe arrival. While simplistic and

imprecise, this sketch demonstrates the inherent need for specialized signals and structures to transmit contractile forces. Contraction requires a road with integrity and adaptability that can meet the harsh demands of muscle contraction.

Contraction's Road from Muscle to Bone: the myotendinous junction

Myotendinous junctions (MTJs) are a section of the road joining muscle and tendon. They are integrated units responsible for transducing contractile forces from muscle to the skeletal system and stabilizing joints. While considering the MTJ, imagine the many stresses muscle and its surrounding tissues are subjected to on a daily basis. The connections that temper and transfer stressful forces without damaging muscle or associated nervous, cardiovascular, and connective tissues are truly functional wonders. Muscle fibers connect to the microenvironment through cell-matrix adhesion complexes. These connections are not static or purely structural. Instead, to translate numerous and stressful forces, sites of cell adhesion at the MTJ are dynamic regions capable of communication and adaption. Collaboration between anchoring complexes and the microenvironment of the MTJ are essential to muscle development and homeostasis.

Inherited muscle diseases known as congenital muscular dystrophies (CMDs) are frequently caused by mutations that weaken attachment between cell-matrix adhesion complexes and the microenvironment. Weakened connections cannot withstand the stress of contraction, and muscle cell detachment, damage and death may result. There is currently no cure for the impaired muscle structure and function exhibited in CMDs. The inherent redundancy and compensation present in cell adhesion are promising resources for developing new treatments. It is therefore an essential project to examine intricacies

underlying how cell adhesion occurs at the MTJ in order to understand how to encourage normal muscle function and homeostasis.

Let's Work Together:the MTJ unit as the whole of many, interactive components

The components responsible for cell-matrix adhesion at the MTJ are arranged in what can be described as "overlapping layers" (Fig. 6). Outermost is the extracellular matrix (ECM). ECM acts as a scaffold for muscle cell attachment and migration. It comprises structural components, ground substance, proteins, and signaling molecules that form a vibrant microenvironment. The structural collagen and elastic fibers give MTJs tensile strength and flexible shape. This scaffold is bathed in a noncellular ground substance that acts as a hub for signaling factors. Proteoglycans and multi-adhesive glycoproteinsreplenish noncellular components, participate in cell-matrix signaling, and facilitate cell adhesion (Ross, 2011; Gilbert, 2003). Proteoglycans consist of proteins andglycosaminoglycans (GAGs). The GAG component attracts water to the ECM to resist compressive forces. The ECM microenvironment is designed to be synergistic; it is in flux to accommodate the needs of muscle tissue. This is not a one-way street though. Cell and matrix participate in bi-directional interaction to influence compositional changes needed to modulate development and muscle maintenance. Though uncertain, it is even projected that the muscle fibers, or cells, themselves are synthesizing and excreting the surrounding ECM material. The interaction between ECM and muscle tissue is known as dynamic reciprocity and is vital to accommodate the complex stresses we incessantly exert on MTJs (Goody and Henry, 2010).

The formation of a basement membrane exemplifies dynamic reciprocity between the ECM and muscle fibers. The basement membrane is a specialized sub-compartment of the ECM and assembles to surround individual muscle fibers. Self-polymerizing laminin glycoproteins and collagen type IV are the primary components forming the continuous basement membrane lining muscle cells. The cross-shaped laminins are heterotrimeric proteins that generate 16 known isoforms from a variable combination of five α, four β, and three γ chains in vertebrates (Goody and Henry, 2008). Chain composition in a laminin isoform has tissue-specific function, and its function can be further specified when laminin receptors are regulated by post-translational events such as glycosylation (Jimenez-Mallebrera, et al., 2004). As a major component of the basement membrane, laminins are linking intermediaries between cell-matrix adhesion receptors and collagen in ECM. Laminin function is not static at the MTJ basement membrane though. Throughout developmental time, different laminin isoforms predominate and dissolve. This occurs as they give cues helping to mediate events in muscle tissue formation (Snow et al., 2008; Peterson and Henry, 2010), such as notochord morphogenesis (Veeman et al., 2008) and cell spreading (Runyan et al, 1988). Laminins are responsible for boundary capture preventing cell elongation past the MTJ (Henry et al., 2005; Snow et al., 2008).

Cell-matrix adhesion is largely dependent on cooperation between laminin and the prominent cell-matrix adhesion complexes the dystrophin-glycoprotein complex (DGC) and receptors of the integrin family. The DGC is a large oligomeric complex. In addition to dystrophin, it is composed of 6 additional membrane-associated proteins. A core component in the DGC is the heavily glycosylated dystroglycan. The extracellular α

subunit of dystroglycan binds to laminin while the integral β subunit forges links to the muscle cell's actin cytoskeleton (Ervasti and Campbell, 1993).

The integrin complexes are a broad class of transmembrane glycoprotein receptors. Important to the purposes of this study are integrin α5β1, α6β1, and α7β1expressed in skeletal muscle tissue. Integrins α5β1 and α6β1 are both present in muscle development yet have distinct roles. Integrin α5β1 is a fibronectin glycoprotein receptor crucial to cell migration during the transition of muscle precursor cells into muscle fibers. While the initial developmental events subside, specifically in the zebrafish, the fibronectin-rich matrix transitions to a laminin-rich basement membrane, and integrin α5β1 disbands its major role as integrins α6β1 and α7β1 persist and gain prevalence. As integrins α6β1 and α7β1 adhere to the ECM via laminin, there is a dynamic, inter- relationship between integrins and ECM components. An intracellular integrin cytoplasmic binding protein (MIBP) interacts with the β1 domain and is expresses during myoblast proliferation to regulate binding affinity for laminin and signal via the intracellular adaptor protein paxillin (Li et al., 2003). Taken together, the transitional presence of integrin heterodimers, corresponding ECM glycoproteins, and bi-directional signaling demonstrate cooperation between cell and ECM. While integrin α7β1 has long been considered the major or exclusive receptor in adult skeletal muscle, recent findings (to be tucked away for more thorough discussion) have elucidated the importance of integrin α6β1 in the establishment of a robust basement membrane (Mayer, 2003; Goody et al., 2012) offering a site for stable adhesion to laminin.

Redundancy in cell-matrix adhesion complexes is a point of great importance in biomedical research. It has been established that dynamic reciprocity occurs at the MTJ.

The ability to compensate and optimize cellular adaption to stress is part of bi-directional signaling. In many inherited muscle diseases, the MTJ is stressed to such an extent that tissues cannot sufficiently adapt and regenerate. This leads to muscle weakness and dysfunction due to toxicity, cell wasting, cell death, and satellite cell dysfunction (Morgan, 2010). This does not mean MTJ components give up as soon as one aspect of normality goes awry. Integrins and the DGC are well-known compensators; if one is compromised the other increases functionality. This intrinsic compensation is a tool. Observing and uncovering compensatory patterns may be exploitedin the development of new therapies.

"Why Me?" Ask Zebrafish: a model system for study of muscle development and disease

To study the intricate redundancies present in muscle tissue and pertinent in disease research, the zebrafish (*Daniorerio)* model organism offers an advantageous paradigm. Despite the fact that teleost fish are more evolutionarily distant from humans than the mammalian mouse model, numerous traits recommend the zebrafish. Transparent embryos, external development, short rearing times, high fecundity, and small size give the model appeal. These characteristics result in easy visualization, large quantity availability, and rapid development conducive to most research. The development of somitic muscle segments are, in fact, so visibly apparent as zebrafish externally develop, they are commonly used as a tool for staging (Kimmel, 1995). In comparison, the *in utero* and slower development in the mouse system is less conducive to this kind of research.

Shared embryonic origin, anatomical and genetic conservation, and the organism's malleability more specifically recommend zebrafish in this type of research. Muscles of the back in mammals are derived from somites, or somitic mesoderm, and have a shared embryonic origin to the segmental skeletal muscle of fish also derived from somites. Somites differentiate into sclerotome, myotome, and dermatome cells that will respectively become vertebrae, muscle, and dermis tissues. There is a higher proportion of myotome than sclerotome in zebrafish somites compared to humans (Devoto, et al., 2006). This allocation results from living in an aquatic environment rather than a terrestrial one. Muscle tissue is more beneficial for movement within low gravity, viscous surroundings. Though dissimilar in this way, plentiful skeletal muscle tissue is present for study. The complexities of cell-adhesion at the MTJ are such that the simplified, plentiful skeletal muscle in zebrafish is preferred over a less straightforward model.

Chevron-shaped boundaries separating muscle segments are analogous to the human MTJ. Human and zebrafish MTJs are each rich in extracellular matrix (ECM) microenvironment, form a basement membrane (BM) sub-compartment, and demonstrate modified laminin isoform and integrin heterodimer content over developmental time (Sztal, et al., 2011) demonstrating consistency with human muscle development. Genetically speaking, orthologous transcripts for all known human dystrophy-causing genesare currently identified in the zebrafish genome (Steffen, 2007). Combined advantages of genetics and simple form allow malleability. Zebrafish can be manipulated as needed using mutant lines, construction of transgenic lines, and reagents to knockdown specified gene-expression calledmorpholinos(Amsterdam and Hopkins, 2006). These

features are particularly attractive for research attempting to elucidate functions in, and to develop treatments for, inherited muscledisease.

Working Towards Treatment: congenital muscular dystrophies (CMDs)

Zebrafish have already been instrumental in research for congenital muscular dystrophies (CMDs). These are a heterogeneous group of inherited diseases characterized by progressive muscle tissue weakness and wasting. As may be expected, some CMDs result when the road between contraction and skeleton fails. Mutations in genes for cell-matrix adhesion receptors and laminin cause CMDs. Those diagnosed experience decline as their musculature is unable to withstand and regenerate from contractile force. Regular stresses become too much for, and overwhelm, compromised musculature. CMDs result in severe debilitation and a drastically shortened lifespan, and there is currently no cure.

Duchenne (DMD) and Becker muscular dystrophies are caused by mutation in the DGC leading to the complete or partial absence of dystrophin. Congenital muscular dystrophy with merosin deficiency is caused a *laminin α2*mutation- essential for major muscle laminin-2 isoform to polymerize (Berger and Currie, 2012). Congenital muscular dystrophy with integrin defect is caused by mutation disrupting the *integrin α7* chain and prevents dimerization of integrin α7β1 (Jimenez-Mallebrera et al., 2005). It is not directly confirmed whether muscle fiber detachment from basement membrane contributes to human CMDs, for biopsies are procured at a safe distance from tendon. MRI studies do suggest more severe damage happens closer to the MTJ (Hasegawa, et al., 1992) and indicate fiber detachment as the instigator of a hostile muscoskeletal environment.

Using the zebrafish model, researchers have shown cell-matrix adhesion failure as the root of muscle cell weakness and apoptosis in several CMDs. Studies have demonstrated and visualized, for the first time, muscle fibers detach prior to cell apoptosis in *laminin α2* (Hall, et al., 2007), *laminin β2* (Jacoby, et al., 2009), and *dag1* (Lin, et al., 2011) mutants. Corresponding to the human system, the *dag1* morphant exhibits a phenotype equivalent to human DMD (Parsons et al., 2002; Berger et al., 2010). Exciting research in a mutant line, demonstrating efficiency of genetic-chemical screens in zebrafish models, uncovered a compound affecting the cAMP-dependent PKA signaling effective in treating dystrophin-deficient diseases (Kawahara et al., 2011). Particularly important to our study, studies using transgenic overexpression as a therapeutic agent have already shown promise. Enhancing transgenic expression of integrin α7β1 to compensate absent dystrophin in DMD mice (Burkin, 2001) and of laminin α2 to partially restore its deficiency in Merosin-deficient mice (Kuang, 1998) models are examples of such efforts. Studies such as these show how powerful the combination of the zebrafish model system, uncovering redundancies, and genetic manipulation is in muscle disease research.

Augmenting Basement Membrane via Nrk2b: a novel pathway for treatingmuscle disease

Cooperation between cell-matrix adhesion complexes and the basement membrane at the MTJ is important to withstand contractile forces. Discontinuity and degeneration of the basement membrane are both signs of and causes of muscle disease. A recent study elucidated a novel pathway in the zebrafish model system, mediated by integrin α6β1, and necessary for basement membrane formation and maintenance.

The nicotinamideriboside kinase (Nrk) 1 and 2 pathways in yeast and human cells are salvage pathways for generating beta Nicotinamide Adenine Dinucleotide (NAD+) via the phosphorylation of a NicotinamideRiboside precursor (Tempel et al., 2007). The previously mentioned MIBP is actually a splice variant of Nrk2. Nrk2 is also a suggested regulator of muscle tissue in these systems. In the zebrafish model, a corresponding Nrk2b ortholog is required for normal muscle growth and development *in vivo* and normal basement membrane assembly at the MTJ (Goody et al., 2010). A strong case was made for NAD+ synthesis as the primary function of Nrk2b (Goody et al., 2010), for both exogenous NAD+ and an NAD+ precursor (in the form of niacin/vitamin B3 found in packaged EmergenC) rescues the *nrk2b* morphant phenotype.

With this knowledge, researchers asked whether exogenous NAD+ would (1) ameliorate abnormal basement membranes and (2), if so, be sufficient to rescue muscle tissue integrity in dystrophic zebrafish. Exogenous NAD+ or EmergenC containing a NAD+ precursor was added at 6 hours post fertilization (hpf). The time frame is of great importance, for it allowed incorporation of exogenous NAD+ before the shield stage can prevent entry of the supplement. In cases of both exogenous NAD+ and EmergenC supplementation, it was sufficient to rescue the dystrophic phenotype and motility in *dag1* or *integrin α7* morphants when accompanied by the *integrin α6β1* receptor (however, from this point forward, the addition of exogenous NAD+ will only be referenced, seeing asEmergenC contains other components in addition to niacin). It was found Nrk2b requires integrin α6β1 to mediate NAD+ synthesis, NAD+ causes localization of paxillin to the MTJ, which leads to increased polymerization of laminin at the basement membrane. How paxillin localization to the MTJ facilitates laminin

organization across the membrane is unknown. Paxillin is an important regulator of cell adhesion turnover; it can both facilitate and degrade cell adhesion.

In a *laminin gamma1* mutant zebrafish, addition of exogenous NAD+ would not rescue dystrophy. Laminin-111 cannot polymerize and form a normal basement membrane in *laminin gamma1* mutants. This suggests the normal basement membrane microenvironment was sufficient for rescuing dystrophy (Goody et al., 2012). While there is still much unknown about *nrk2b* expression, these findings hold therapeutic potential the *nrk2b* pathway and augmentation of the basement membrane.

Glitches with NAD+ Supplementation as a Therapy

While these studies have made astonishing strides, NAD+ supplementation is still far from being a solution for human muscle disease. Three difficulties present immediate caveats. (1) While comparative analysis of zebrafish and human genomes show conserved gene function, their systems are separated by 450 million years of evolutionary departure (Posteltwait et al., 2000). There may be conservation between the zebrafish *nrk2b*and the human *nrk2*, but these pathways are also functioning in different systems with a long history of divergence. In short, what occurs in the zebrafish via nrk2b-mediated NAD+ supplementation may be very different from what occurs in humans.

(2) Treatment administration does not correlate between zebrafish and human. Human muscle diseases, such as congenital muscular dystrophies, are typically not diagnosed until after birth. If a young boy is diagnosed with DMD, will the alleviating effects of NAD+ supplementation be enough tohelp in light of the extremity of his condition? So far, studies have begun treating zebrafish at developmental stages

equivalent to early *in utero*human development. Whether the results are replicable in more mature animals is an important question moving forward. NAD+ may not be able to sufficiently augment basement membrane in older models *after* early development and structural establishment of the ECM and basement membrane.

(3) CMDs are very severe forms of muscle disease. NAD+ supplementation has been shown to reduce dystrophy, but will that be enough to help those with the severest types of muscular dystrophy? The studies showed muscle fiber detachment rates decreased, MTJ structure was amended, and motility was improved with vitamin supplementation. It comes down to whether NAD+ supplementation can bolster the MTJ microenvironment to a high enough degree to prevent toxicity and inability to stably regenerate muscle tissue.

While supplementing NAD+ via a vitamin precursor holds promising outlooks for future research, there are many uncertainties present. NAD+ supplementation currently does not translate into an effective treatment for a human child diagnosed with DMD.

A Case for Nrk2b Overexpression

The nrk2 pathway can be therapeutically exploited differently from vitamin supplementation. Gene therapy is an important area of research focused on alleviating inherited diseases. Gene therapies are attractive, for the treatment is targeted in a tissue specific manner. In diseases that are often the result of single gene mutation, this specificity provides a more direct route, whereas NAD+ supplementation through the blood stream would not be utilized by muscle tissue alone. As mentioned, studies using transgenic overexpression as a therapy have shown promise. Enhancing transgenic

expression of integrin α7β1 to compensate absent dystrophin in DMD mice (Burkin, 2001) and of laminin α2 to partially restore its deficiency in Merosin-deficient mice (Kuang, 1998). Is nrk2b gene therapy a potentially better treatment for CMDs than NAD+ supplementation?

The purpose of this study is to begin answering this question. So, we asked if *nrk2b* overexpression was sufficient to rescue muscular dystrophy in *dag1* morphants. We next hypothesized NAD+ supplementation and *nrk2b* overexpression shouldessentially have the same effect, so far as is known. The primary role of both is to increase NAD+ levels already shown to alleviate muscle form and function in *dag1* morphants. We additionally hypothesized overexpressing *nrk2b* will create a greater potential for the organism to compensate for the missing DGC. It will do so by cell autonomous paxillin localization and cell non-autonomous laminin polymerization at the basement membrane. Taking all into consideration, we hypothesized overexpression of *nrk2b* via a transgenic construct would be sufficient to alleviate dystrophy in *dag1* morphants.

Exploiting the novel *nrk2b* pathway for gene therapy in *dag1* morphant zebrafish has potential. As a more direct therapeutic option than supplementation, finding a viable mode of mimicking*nrk2b* overexpression may be more effective in the human system. We constructed an overexpressing *nrk2b* transgenic line. We detected the integrated *nrk2b* genes in the zebrafish when strong GFP expression occurred in F_0generation fish. Only these fish were raised to an age at which they could spawn. Breeding pairs that spawned consistent, glowing GFP F_1generation fish were kept. We utilized F_1generation fish stably expressing GFP to evaluate the therapeutic potential of *nrk2b* overexpression.

In this study, we were interested in (1) characterization of the novel *nrk2b* overexpressing zebrafish phenotype and (2) determining whether overexpression is sufficient to alleviate dystrophy when injected with *dag1* morpholino. We hypothesized that a normal zebrafish phenotype would occur in the transgenic zebrafish and that *nrk2b* would be sufficient to rescue *dag1* induced dystrophy. Muscle tissue and MTJs were characterized in *nrk2b* transgenic controls and in *nrk2b* transgenic/*dag1* morphants. Findings did not support our hypothesis. We observed abnormal MTJ boundaries and delayed development in *nrk2b* transgenic controls. Instead of ameliorating dystrophy in *dag1* morphants, the overexpressing *nrk2b* transgenic fish showed abnormally wide MTJ angles and detached fibers. These data tentatively suggest *nrk2b* transgenic zebrafish are inherently abnormal, and *nrk2b* overexpression is not sufficient to alleviate dystrophy.

Methods

Zebrafish (Daniorerio) husbandry

Adult zebrafish were kept at 28.5^{o}C on a 16 hour light and 8 hour dark cycle. I collected zebrafish embryos from the natural spawning of these adult fish and staged embryos in accordance with Kimmel et al. (1995). All fish were treated and maintained in accordance with the University of Maine IACUC.

Transgenic lines

A transgenic line expressing GFP fused to the C-terminus of Nrk2b was generated by Michelle Goody and Mary Astumian of the Henry lab by cloning full length Nrk2b from 24 hours post fertilization (hpf) zebrafish cDNAthat was prepared as described in ZFIN:Molecular Methods. The PCR product was cloned into the pDONR221 plasmid (Invitrogen). The resulting pME, or "middle" clone, was cloned, along with p5E-bactin2 (a non-specific beta-actin promoter- a 5' entry vector from Tol2Kit (Kwan, 2007)) and p3E-EGFPpA (EGFP for C-terminal fusions, plus SV40 late polyA- a 3' entry clone vector from the Tol2Kit (Kwan, 2007)), via a recombination reaction. Linearized pCS2-TP plasmid (provided by Koichi Kawakami) was used as a template to generate capped mRNA encoding Tol2 transposase by *in vitro* transcription using SP6 polymerase (Message Machine, Ambion). Plasmids were co-injected with mRNA encoding Tol2 transposase into wildtype AB embryos at the 1-cell stage using a MPPI-2 Pressure Injector from ASI. GFP expressing zebrafish from the F_o generation were grown to maturity and spawned to detect for germ line integration of the transgene. Those of the

F_1generation, the offspring of the F_o generation with germ line integration of the transgene, stably overexpressing Nrk2b were used for experimentation.

Morpholino (MO) injections

MOs are used to knockdown gene expression through partially blocking cytosolic initiation of translation or modify pre-mRNA splicing in the nucleus. I used an antisense morpholino oligonucleotide to partly block *dystroglycan* translation initiation obtained from Gene Tools, Inc. The sequence used was 5'-CATGCCTGCTTTTATTTTCCCTCGC-3'. It was the same as designed and microinjected according to Parsons et al. (2002). I injected a volume of 1.4nl into the yolk of 1-cell stage embryos to deliver 7ng of *dystroglycan* MO using a MPPI-2 Pressure Injector from ASI. The morpholino solution I made was a 1:15 dilution of dystroglycan (5'-CATGCCTGCTTTTATTTTCCCTCGC-3') in sterile water.

Electrical Stimulation

I electrically stimulated the *dag1* morphant and control Nrk2b transgenic zebrafish at 72 hours post fertilization using Grass SD9K-Square Pulse stimulator. This step was taken to induce depolarization of the muscle membrane and cause a stressful contraction. Normal muscle should be able to withstand this stress while dystrophic muscle would not withstand this stress. The stimulator was set at 30V, 8msec duration, 4 pulse/secfrequency, and 6msec delay. Zebrafish were anesthetized in a tricaine solution (1:125 tricaine in embryo rearing medium) then stimulated for 1 minute per fish by

positioning the electrode in a head-to-tail direction. I fixed fish in 4% paraformaldehyde directly they were stimulated.

Fixation

I put embryos undergoing fixation in tubes with 4% Paraformaldehyde (PFA) for 4 hours at room temperature or overnight at 4^{o}C on their sides. Fixed embryos were washed 5 times for 5 minutes (5x5) in decreasing volumes of 0.1% PBS-Tween.

Phallodin staining

My staining of the fish with Alexa Flour 488 (Molecular Probes) phallodin involved a series of steps: permeabilizing embryos for 1.5 hours in 2% PBS-Trition, washing 5x5 in 0.1% PBS-Tween, incubation in a 1:20 dilution of in 0.1% PBS-Tween overnight at 4^{o}C, washing 5x5 in 0.1% PBS-Tween, and storage in 0.1% PBS-Tween.

Mounting

Deyolking occurred in PBS using insect pins under a compound microscope. They were transferred to 80% glycerol for 5 minutes, side mounted on slides, sealed with cover slides, and stored in light-protective cases at 4^{o}C.

Imaging

All imaging was completed on a Zeiss Axio Imager Z1 microscope with a Zeiss Apotome attachment. Slides were viewed under the 10x, 20x, or 40x objective. The filter channels

38HE (GFP) and 43HE (DsRed) were respectively used to image phallodin and GFP. Z-stacks were taken in incremental slices of 1.5 µm.

Measuring and analyzing myotome boundary angles

I measured and calculates the angles of the chevron shaped myotenidinous junctions on phallodin and GFP stained images using Zeiss Inside 4D software. The angles were averaged within conditions of treatment. Averages were graphed and statistically analyzed for significant abnormalities compared to the MTJS angles of wildtype zebrafish.

Results

nrk2b transgenic MTJ boundary angles are significantly wider than wildtype control MTJ boundary angles.

Since the *nrk2b* overexpressing transgenic is a novel transgenic zebrafish and has not been characterized, the first question we asked was if the *nrk2b* transgenic phenotype was normal. Deleterious effects of *nrk2b* overexpression would not be conducive to a gene therapy approach. To investigate, we spawned the transgenic fish strongly expressing GFP for three rounds of experimentation and simultaneously raised wildtype controls. Each round, they were grown for 72 hours, because the *dag1* morphants' dystrophic phenotype typically occurs at this time. At this time, they were electrically stressed with an electrode to test for fiber attachment integrity. We next fixed, stained, mounted, and imaged the specimens (reference pg. 14 for more specific **Methods**). We used phallodin to stain actin to visualize musculature. Muscle tissue mass, striations, motility and fiber adhesion to MTJ boundaries appeared normal in *nrk2b* transgenics at 72 hpf. Fiber detachment was present in three of twenty embryos (Fig. 5D, 4.61±14.04 percent myotomes per embryo). However, due to the majority having no detachment, we speculate these are outliers.

The *nrk2b* transgenics were developmentally delayed. At 22 hpf, *nrk2b* transgenics consistently exhibited 22 somites rather than 26 somites. Another characteristic indicative of developmental disruption, the *nrk2b* transgenics also exhibited abnormally wide MTJ boundary angles.

Using Zeiss Inside 4D software, we measured *nrk2b* transgenic and wildtype MTJ angles. In 72 hpf zebrafish, we determined chevron-shaped angles were significantly

wider in *nrk2b* transgenics (Fig. 1B) compared to wildtype zebrafish (Fig. 1A) at 72 hpf(Fig. 1C, average MTJ angle for *nrk2b* transgenic controls, 103.41±8.65 degrees, $p<0.05$). This phenotype characteristic of *nrk2b* overexpression was reminiscent of *nrk2b* morphants. In Goody et al. 2010, *nrk2b* morphants, deficient in Nrk2b, also exhibited wide MTJ angles (Fig. 1C average MTJ angle for *nrk2b* morphants, 118.1027±6.84). 100 µm of exogenous NAD+ rescued the *nrk2b* morphant phenotype (Fig. 1C, average MTJ angle for *nrk2b* morphants + 100µm NAD+, 97.6611±8.30), indicating the primary role of *nrk2b* was NAD+ synthesis.

The *nrk2b* morphants are likely Nrk2b deficient. This contrasts with the effect of *nrk2b* overexpression, yet the phenotypes of *nrk2b* morphant and *nrk2b* overexpressing zebrafish have striking similarities. The wide angle phenotype is interesting, for it shows comparable effects of abnormally low and high gene expressions. This is contradictory to previous studies examining high and low expressions. Previous studies show contradictory effects of both low and high expression. In a study examining the role of mutated integrin α6 in prostate cancer, data indicate preventing integrin α6 cleavage will rescue bone loss and pain, but expression of cleavable integrin α6 results in increased bone loss and pain (King et al., 2008). How *nrk2b* high and low expression generates wide angles is an area for further research.

MTJ boundaries are intact in nrk2b transgenic zebrafish.

To investigate similarities between the *nrk2b* transgenic and *nrk2b* morphant phenotypes, we quantified boundary crossings on images. Wildtype zebrafish have fibers attached to continuous MTJs at each myotome (Fig. 2A; Fig. 2C, in wildtype controls

0/282 or 0 percent MTJs were crossed). Discontinuous MTJs and muscle fibers extending across the MTJ are a hallmark of a disrupted MTJ. In *nrk2b* morphants, wide angles are accompanied by significant boundary crossings (Goody et al, 2010; Figure 2E, in *nrk2b* morphants 18/87 or 21.42 percent of MTJ were crossed). Along with wide angles, boundary crossings were rescued by adding exogenous NAD+ (Goody et al, 2010; Fig. 2E, in *nrk2b* morphants 6/93 or 6.45 percent of MTJ were crossed). The *nrk2b* transgenic MTJs were all intact (Fig. 2B; Fig. 2E, in *nrk2b* transgenics 0/201 MTJs were crossed). Interestingly, while*nrk2b* morphants exhibit crossings, the zebrafish overexpressing *nrk2b* have no boundary crossings. Taken together, these data suggest wide angles and boundary crossings can be mutually exclusive characteristics.

nrk2b transgenic/dag1 morphant MTJ boundary angles are significantly wider than wildtype control and nrk2b transgenic MTJ boundary angles.

We made fish that were both Dag1-defiecient and *nrk2b* overexpressing to test whether the dystrophic phenotype of *dag1* morphants was alleviated by the *nrk2b* overexpression. We characterized the phenotype of these zebrafish to evaluate whether *nrk2b* overexpression would be a viable form of gene therapy for a congenital muscular dystrophy model.

Occurrence of wide angles was inspected in *nrk2b* transgenic*/dag1* morphants. We injected *dag1* morpholinos to create Dag1-deficient *nrk2b* transgenic zebrafish. These fish were also grown until 72 hpf, electrically shocked, and prepared for imaging as previously described. Wide angles were maintained in the*nrk2b* transgenic*/dag1* morphant phenotype (Fig.3C). Since wide angles are not characteristic of the wildtype

injected *dag1* morphant, it follows that they are a consequence of *nrk2b* overexpression. Using angle measurements, we found the *nrk2b* transgenic*/dag1* morphant MTJ angles were significantly wider than both wildtype and *nrk2b* transgenic controls (Fig. 3D, average MTJ angle for *nrk2b* transgenic/*dag1* morphants, 106.78±9.55 degrees, $p<0.01$ compared to wildtype, $p<0.05$ compared to *nrk2b* transgenic control). Exacerbated angles in *nrk2b* transgenic/*dag1* morphants compared to *nrk2b* transgenic controls suggest Dag1 and *nrk2b* expression have a synergistic relationship during the formation of normal boundary angles.

MTJ boundaries are intact in nrk2b transgenics/dag1 morphant zebrafish.

To investigate whether continuity at the MTJ is maintained with a Dag1-deficientcy, we examined the boundary crossings in *nrk2b* transgenics*/dag1* morphantzebrafish.. The *nrk2b* transgenics*/dag1* morphant, like the *nrk2b* transgenic, has no boundary crossings and MTJs appear fully intact (Fig, 4A-D, in *nrk2b* transgenic/*dag1* morphants 0/57 or 0% were crossed).

Significant fiber detachment in nrk2b transgenic/dag1 morphants

We had initially asked if *nrk2b* overexpression in *dag1* morphants would be sufficient to rescue dystrophy. Injection of the *dag1* morpholino in wildtypes results in a phenotype reminiscent of human DMD with significant fiber detachment by 72 hpf (Panzer et al., 2002;Berger et al., 2010). Given that NAD+ supplementation rescues *dag1* morphant fiber detachment, and the primary role of *nrk2b* is thought to be NAD+ synthesis, we hypothesized that it seems *nrk2b* overexpression should rescue fiber

detachment. Taken together, the *dag1* morphant + 100µm NAD+ and the *nrk2b* transgenic*/dag1* morphants should theoretically have similar phenotypes. We visualized fibers in *nrk2b* transgenic*/dag1* morphants and found the majority of somites had detached fibers (Fig. 5C-D, 67.44±16.84 percent disrupted myotomes per embryo). *Nrk2b* overexpression thus did not alleviate dystrophy in *dag1* morphants. If anything, dystrophy was more severe in the *nrk2b* transgenic*/dag1* morphants than in just *dag1* morphants. Rather than rescuing dystrophy, *nrk2b* overexpression appears to exacerbate dystrophy in *dystroglycan* deficient fish.

Discussion

Significance of Research

Duchenne, Becker, Merosin-deficient, Integrin-deficient, and Fukuyama are congenital muscular dystrophies disrupting normal cell-matrix interactions at the MTJ (Berger, 2010; Jimenez-Mallebrera, 2004; Lin, 2011). These are debilitating and lethal diseases with no current cure. Gene therapy, a relatively new area of study, has received attention as a potential treatment method for congenital muscular dystrophies. Clinical trials using viral and non-viral gene therapy methods are ongoing (Brooker, 2011), and the first human clinical trial for the most frequently occurring congenital muscular dystrophy, Duchenne muscular dystrophy, began in 2006. Following this trial, three more immediate trials were planned (Foster et al., 2006). This indicates that the trial broadened the possibilities and knowledge of how gene therapy may become a viable treatment option. However, there is still no current cure. Due the difficulties in replacing or repairing a defected gene and the multifaceted nature of the severe symptoms, research requires more time and efforts. Human gene therapy trials result from intensive study of model organisms. Another promising route may be using the Nrk2b kinase as a pharmacological target. Activating the kinase, rather than maneuvering the complexities of delivering a gene, is tissue specific and is an emerging therapeutic tactic. With zebrafish at our fingertips, genetically and anatomically suited to the of study inherited muscle diseases, we are in a prime position to research prospective gene therapies and kinases.

Here we are interested in *nrk2b* gene overexpression as a possible therapy for multiple congenital muscular dystrophies arising at the MTJ. In this novel salvage

pathway, Nrk2b mediates NAD+ synthesis. The pathway uses α7β1and requires integrin α6β1 for NAD+ to improve the basement membrane in zebrafish (Goody et al., 2010; Li et al., 2003; Goody et al., 2012). NAD+ potentiates paxillin localization and laminin polymerization to augment the MTJ microenvironment for fiber attachment. Addition of 100µm exogenous NAD+ has already been shown to reduce dystrophy in *dag1* morphants (Goody et al., 2012).

Research suggests NAD+ synthesis is the primary role of *nrk2b* (Goody et al, 2010). Hence, manipulation of *nrk2b* expression will affect NAD+ synthesis without off-target effects. Taken together, *nrk2b* overexpression in a *dag1* morphant is theoretically equivalent to administering exogenous NAD+ to a *dag1* morphant. Gene therapy, however, is a more precise mode of treatment, for it has the advantage of being tissue specific. We therefore researched whether *nrk2b* overexpression is sufficient to rescue dystrophy in the Dag1-deficient, congenital muscular dystrophy model.

Characterization of nrk2b Overexpression in the Transgenic Control: wide MTJ angles

Gene expression should be manipulated with extreme care. Examining the *nrk2b* overexpressing phenotype is necessary to detect whether abnormalities are caused by this particular genetic manipulation. Wide MTJ angles observed suggest developmental processes are disrupted by *nrk2b* overexpression. Wildtype zebrafish normally form chevron-shaped MTJs (Figure 1A**).** The V-shaped angle of the MTJ boundary is indicative of normal muscle fiber arrangement required to generate propulsive forces while swimming (Currie and Ingham, 2001). Complex interactions lead to segmentation with normal fibers and MTJ angles (Kimmel et al, 1995; Currie and Ingham, 2001).

Myotomes are initially grouped, cube-shaped precursor cells delineated by early MTJs oriented at wide angles (Fig. 7). The chevron-shaped angle forms as the muscle precursor cells striate and elongate into multinucleate myotubes. In an event known as boundary capture, the MTJ boundary components trap fibers and prevent them from extending past the MTJ (Henry et al., 2005). Since wide angles are present in *nrk2b* overexpressing fish, segmentation is somehow disrupted.

If the primary role of *nrk2b* expression is to mediate NAD+ synthesis, why is *nrk2b* overexpression causing wide boundary angles when adding pure, exogenous NAD+ does not? Perhaps an obvious answer is that NAD+ synthesis might not be the singular role of *nrk2b* expression. *nrk2b*expression may have additional effects of which we are currently unaware. To be pragmatic, speculating the many possible other effects *nrk2b* expression may have is outside the scope of this research.

This study can however address the 3-hour time gap between *nrk2b* expression andNAD+ supplementation. In Goody et al., exogenous NAD+ was added at 6 hpf. In contrast, the *nrk2b* transgene is a zygotic gene, and the maternal-to-zygotic transition (MZT) initiates its expression. MZT is a process by which zygotic genes are expressed for the first time as zygotic transcript activation turns on and maternal transcripts degrade. The first major wave of this MZT in zebrafish occurs at 3 hpf (Tadros et al., 2009). This leaves a 3 hour window between 3 hpf when *nrk2b* zygoticoverexpression first occurs and addition of exogenous NAD+ at 6 hpf to treat zebrafish.

The *nrk2b* transgene is being expressed between the 512-cell and 1k-cell stages in the blastula period until shield stage in gastrulation all before NAD+ addition (Kimmel, 1995). Critical events occur at this time. Gastrulation is a period of complex cell

rearrangements allowing a sheet of cells to transition, according to cell fate, into a three dimensional embryo. Of particular importance to our study are the cell movements, known as convergence extension, that shape embryos to become longer along the anterior-posterior axis and narrower across the mediolateral axis (Fig. 7). Convergence extension, more succinctly put, is a medio-lateral intercalculation important to body axis elongation (Wolpert, 2007). During convergence extension, cells migrate towards an anterior-posterior axis. As more cells arrive, or "converge," at the so-called axis, they align in a way that elongates the axis. These medio-lateral intercalculation movements are reminiscent of a group of children surging forward to gather in a straight line for the school bus (Fig. 7). The active movements are powered by cell polarity. Polarity generates lamellipodia that extend cell borders, participating in cell-matrix adhesion turn-over, to direct cell migration (Wolpert, 2007). Defects in these movements, occurring in the 3 hour time window, may cause the wide MTJ angle phenotype seen in *nrk2b* transgenics.

A rigorous investigation of this 3-hour window is important in future research. Using *nrk2b* transgenics overexpressing *nrk2b* under control of the heat shock promoter, we could induce gene overexpression at 6hpf, the time of exogenous NAD+ addition. We could compare the phenotype when *nrk2b* overexpression was induced at 6hpf compared to 3 hpf. Comparing and contrastingof phenotypes would elucidate the affect *nrk2b* expression in this space of developmental time.

Comparing the *nrk2b* morphant phenotype with the *nrk2b* overexpressing phenotype, we found the wide angle abnormality is common in both high and low expressions of *nrk2b*. As discussed, this is of particular interest simply because high and

low expressions of nrk2b are inverses. It follows that they would have opposite effects. As the study on the role of integrin α6 in bone wasting and pain in mice suggests, high expression and low expression will have different outcomes (King et al., 2008). In this study, the same abnormality arises from over- and under-expressed *nrk2b.* These data suggest there is a balance to be had when it comes to *nrk2b* expression. There is an intrinsic 'just-right' amount and extremes on either end are detrimental to some of the same mechanisms. As it has been described in other fields, it falls under the 'Goldilocks Principle.'

While discussing extreme and 'just-right' amounts, it is important to acknowledge the dosage response issue in this research. In Goody et al., different doses of exogenous NAD+ were administered to detect the levels of supplementation sufficient to treat or be toxic to zebrafish. While there are ways to determinethelevels of NAD+ generated in excess of wildtype levels in *nrk2b*transgenics, it is outside the scope of this study to do so. Although the transgenics utilized in this research stably expressed GFP, we do not know how frequently the transgene integrated into the genome. Although an incomplete test of dosage, an important experiment to perform to determine the occurrence and frequency of *nrk2b* integration in the genome is a Southern blot. At this time, however, there is no complementary DNA probe. An in situ hybridization (ISH) is a second option to determine the presence of mRNA for Nrk2b in the *nrk2b* transgenic compared to wildtype zebrafish.

Characterization of nrk2b Overexpression in the Transgenic Control: boundary crossings

The average wide MTJ angle is significantly larger in the *nrk2b* morphant than in the *nrk2b* trangenic zebrafish ($p<0.01$). Boundary crossings are also typical of the *nrk2b* morphant phenotype yet are absent in the *nrk2b* transgenics. As mentioned, this indicates boundary crossings and wide angles can occur separately. Complicating this observation is the knowledge that supplementing *nrk2b* morphants with exogenous NAD+ alleviates both the boundary crossings and the wide angle phenotype. Taken together, this indicates that while wide angles and boundary crossings can occur separately, if they occur at the same time, the defects may exacerbate the phenotype.

Lack of boundary crossings in the *nrk2b* transgenic suggests that the basement membrane is continuous. An intact basement membrane is indicative of sufficient laminin polymerization for capturing fibers at the MTJ. It has been determined*nrk2b* is non-cell autonomously required for laminin polymerization at the basement membrane (Goody et al., 2010). Laminin isoforms also influence various developmental stages like normal fast-twitch muscle fiber elongation (Snow et al., 2008). The *nrk2b* morphants were stained for laminin, and there were gaps throughout the basement membrane where laminin had not polymerized (Goody et al., 2010). Taken together, the data suggest *nrk2b* transgenics have sufficient laminin polymerization to form a continuous boundary that captures muscle fibers. This makes logical sense, for *nrk2b* transgenics should be synthesizing more NAD+ than both *nrk2b* morphants and wildtypes to potentiate laminin polymerization at the basement membrane. To investigate these observations further, we would next stain for laminin-111 in the *nrk2b* transgenics. It would be interesting if

laminin organization at the basement membrane was increased in *nrk2b* overexpressing fish compared to wildtypes. This might indicate that excess laminin polymerization is possibly detrimental.

Characterization of nrk2b Overexpression in the dag1 Morphant: wide MTJ angles and boundary crossings

We next examined the phenotype *nrk2b* overexpression in a *dag1* morphant. Initially, we hypothesized the *nrk2b* overexpression would be equivalent to NAD+ supplementation. Meaning, the *nrk2b* overexpression would augment the basement membrane to improve the microenvironment to increase fiber attachment integrity. The unexpected *nrk2b* transgenic control phenotype, however, indicated the dystrophy in the *dag1* morphant may not be rescued if the MTJ microenvironment was compromised prior to the augmenting effects of NAD+.

The wide MTJ angles persisted in the *dag1* morphant. The angles were even significantly wider than in the *nrk2b* transgenic control. This indicates further disruption at the MTJ, such as in Dystroglycan deficiency, may worsen wide MTJ angles. As we previously observed, wide angles in the *nrk2b* morphants were possibly exacerbated by the additional characteristic of insufficient laminin polymerization. Taken together, these observations reinforce that the interdependent interactions occurring between the components maintaining the MTJ integrity are complex.

Boundary continuity was next examined in the *nrk2b* overexpressing *dag1* morphants, and no boundary crossings were observed. This indicates laminin polymerization was still sufficient, even with Dag1-deficiency, to maintain boundary

capture. However, as we would in the *nrk2b* transgenic controls, it is important to investigate more thoroughly by staining for laminin-111. This would show whether the basement membrane is continuous in this condition.

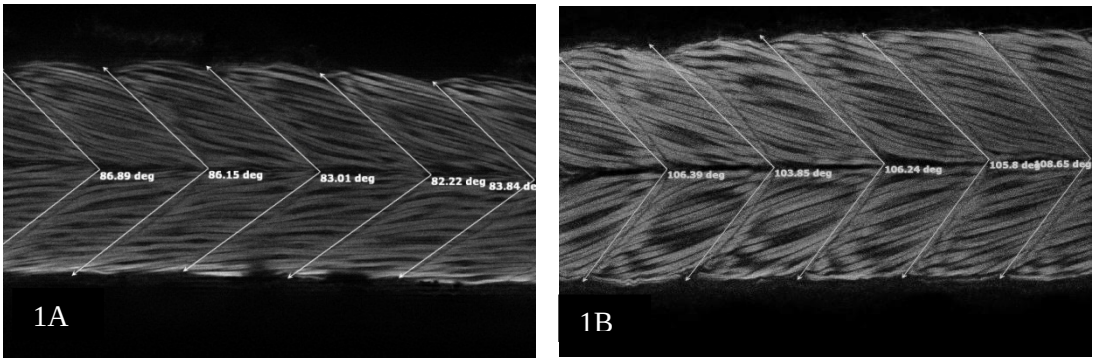
Fiber Detachment in nrk2b Overexpressing/dag1 Morphants

With *nrk2b* overexpression, significant fiber detachment occurred in the *dag* morphant. In comparison to previous studies, the fiber detachment was more extreme in *nrk2b* overexpressing fish than in wildtype zebrafish injected with *dag1* morpholino as well as Dag-deficient zebrafish treated with 100μm of exogenous NAD+. This may indicate the *nrk2b* overexpression and wide angles increased the normal amount of dystrophy seen in a *dag1* morphant. However, there are several uncertainties to consider: the dosage of NAD+ generated in *nrk2b* transgenics, the discrepancies between morpholinos, and the electrical shock we stressed the zebrafish with to test dystrophy. Each of these may have increased (or decreased) the amount of fiber detachment observed in the *nrk2b* overexpressing*/dag1* morphants. However, we suspect the MTJ microenvironment was already not augmented sufficiently to rescue fiber detachment. While NAD+ supplementation is sufficient to promote the inherent compensation by integrins, in the absence of dystroglycan, by mediating laminin polymerization and paxillin localization to organize the basement membrane, *nrk2b* overexpression was unable to sufficiently influence formation of a microenvironment conducive to fiber attachment.

Conclusion: final thoughts

We approached this project to determine whether *nrk2b* overexpression in transgenic zebrafish was sufficient to rescue dystrophy in a congenital muscular dystrophy model. Instead we opened a can of questions regarding the role of *nrk2b* overexpression. The wide MTJ angle phenotype and intact boundaries demonstrate boundary crossings and wide angles are separate characteristics in the zebrafish phenotype. Comparable wide angles in *nrk2b* morphants and *nrk2b* transgenics further suggest that there exists a just right amount expression of *nrk2b*. The just-right amount, when many gene therapies focus on increasing or decreasing expression, is important to take into consideration in future studies adjusting *nrk2b* expression. Both the *nrk2b* transgenic control and *nrk2b* transgenic *dag1* morphant phenotypes reveal there is a great deal unknown about *nrk2b* expression, and it must be more fully elucidated before it is considered as a route for gene therapy.

Figure 1



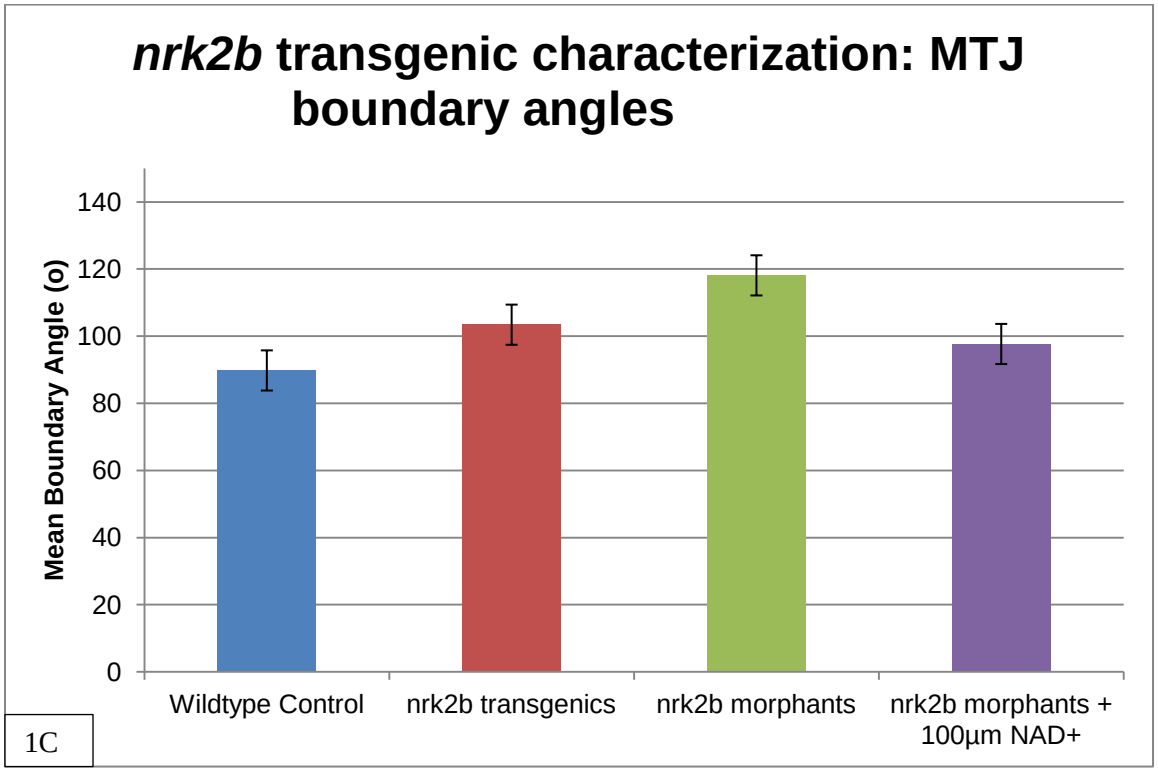
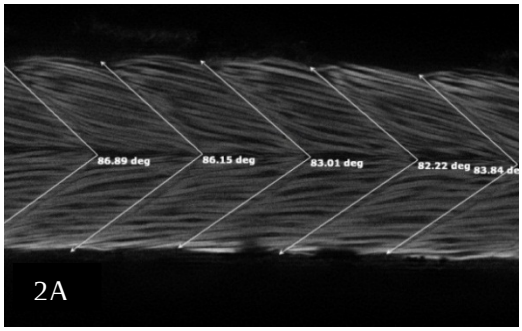
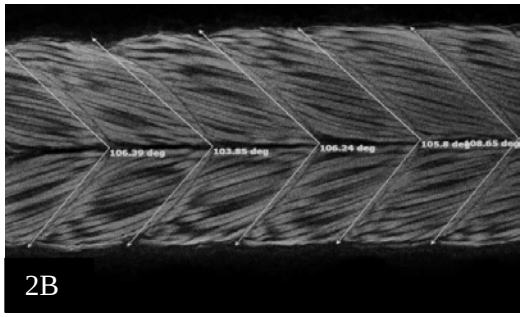


Figure 1. The *nrk2b* overexpressing transgenic zebrafish imaged, side mounted, and phallodin stained with MTJ angle measurements at 72 hpf. (A) AB wildtype control with normal MTJ angle measurements depicted. (B) A*nrk2b* overexpressing transgenic with the significantly wider MTJ angles. (C) Graph additionally demonstrates the wide angle phenotype in *nrk2b* overexpressing transgenics and *nrk2b* morphants (images not shown). The *nrk2b* morphant wide angle phenotype is significantly rescued by NAD+ supplementation (*nrk2b* morphant/NAD+ data from Goody et al., 2010). These results demonstrate similar wide MTJ angles in both *nrk2b* overexpressing transgenic and *nrk2b* morphant phenotypes. These results suggest a 'just-right' amount of *nrk2b* gene expression might occur. Error bars show standard error.

Figure 2





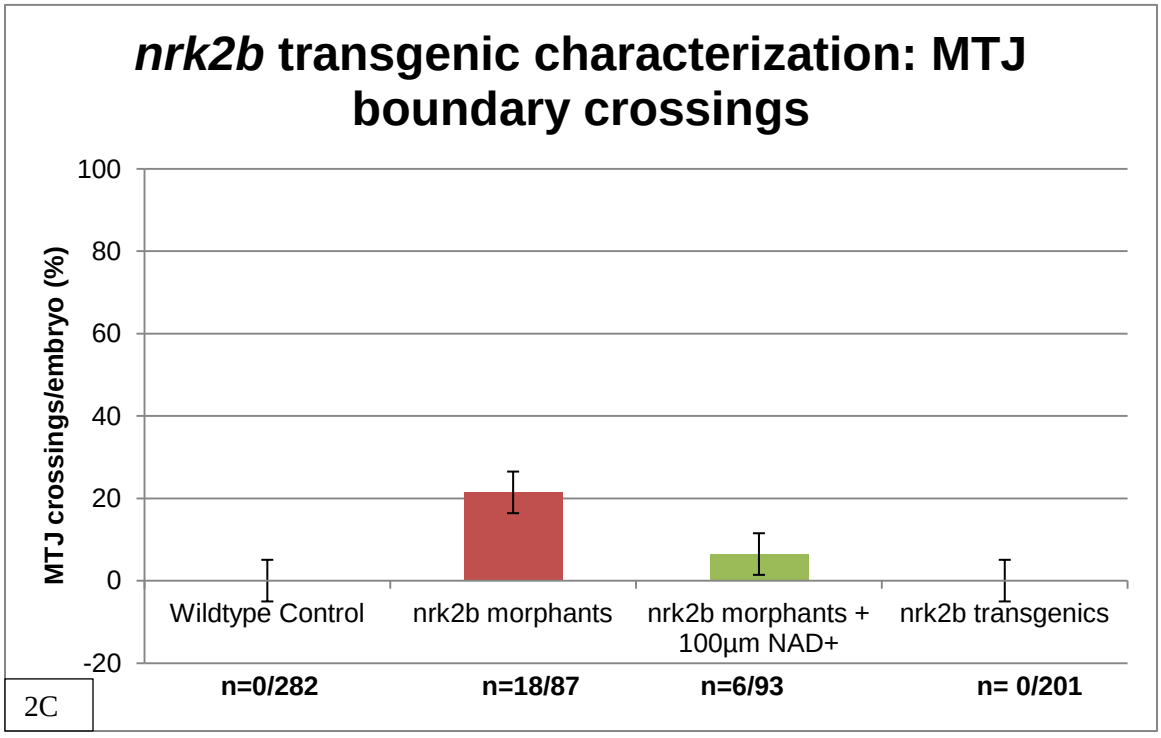
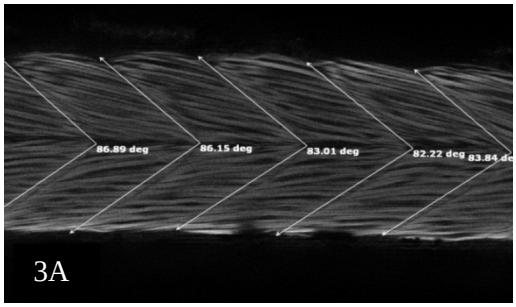
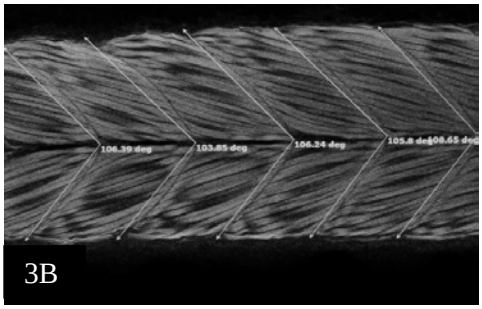
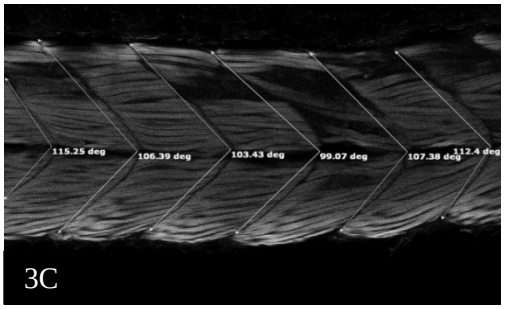


Figure 2. The MTJ boundaries exhibit no boundary crossings in *nrk2b* transgenic zebrafish imaged, side mounted, and phallodin stained with MTJ angle measurements at 72 hpf. (A) AB wildtype control with no boundary crossings across the MTJs. (B) A *nrk2b* overexpressing transgenic with no boundary crossings across the MTJs. (C) The graph shows that, while boundary crossings don't occur in *nrk2b* overexpressing transgenics, significant boundary crossings occur in the *nrk2b* morphants (images not shown). Boundary crossings are rescued in *nrk2b* morphants when supplemented with NAD+ (*nrk2b* morphant/NAD+ data from Goody et al., 2010). These data suggest the wide MTJ angle and boundary crossings are distinct phenotypic characteristics.

Figure 3







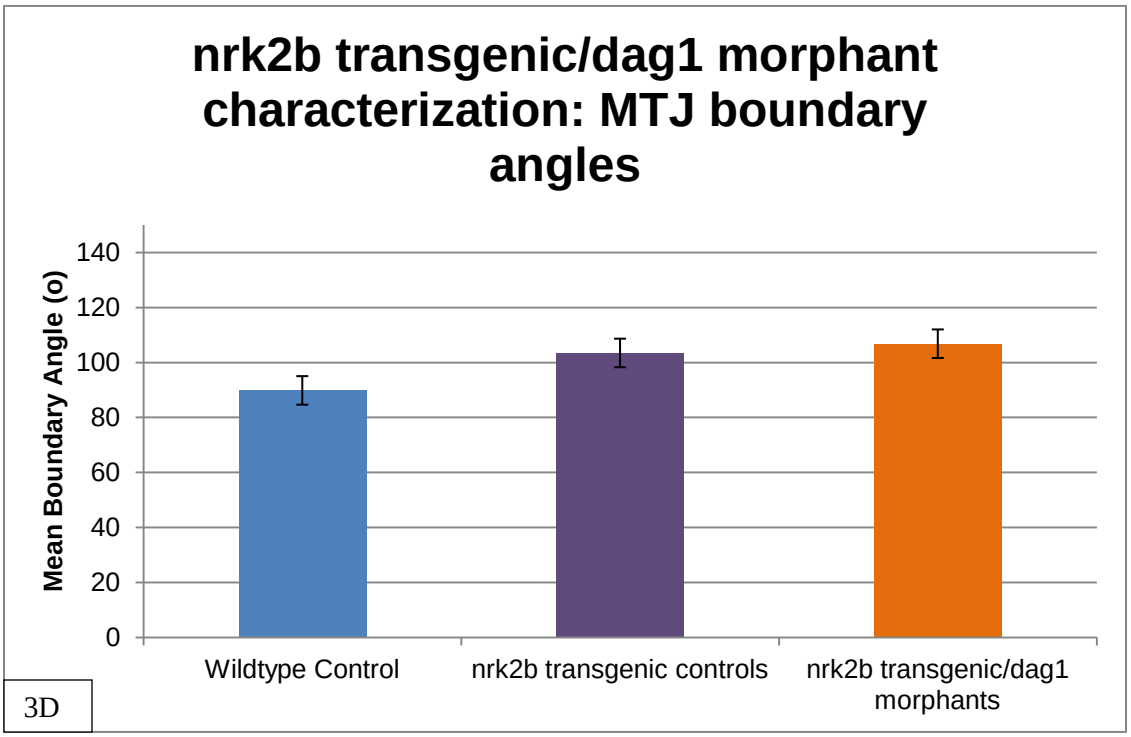
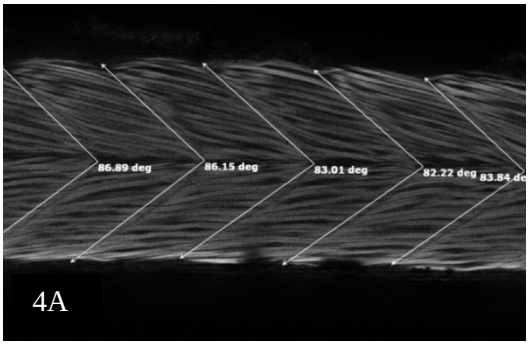
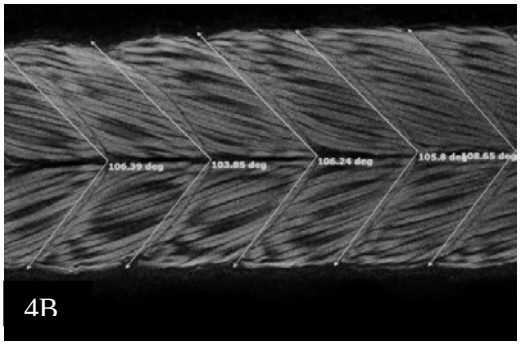
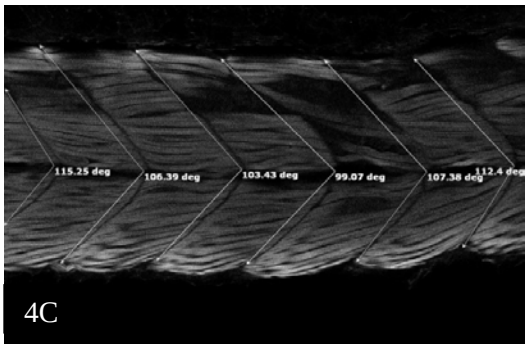


Figure 3. The *nrk2b* transgenic*/dag1* morphant has significantly wider MTJ angles than *nrk2b* transgenics. Zebrafish are imaged, side mounted, and phallodin stained with MTJ angle measurements at 72 hpf. (A) AB wildtype control with normal MTJ angle measurements. (B) A*nrk2b* overexpressing transgenic with abnormal, wide MTJ angles. (C) A *nrk2b* transgenic*/dag1* morphant with significantly wider MTJ angles than the *nrk2b* overexpressing transgenic. (D) Graph shows the wide MTJ angles are exacerbated in Dag1-deficient fish. These data suggest *dag1* and *nrk2b* expression synergistically cause normal MTJ angles.

Figure 4





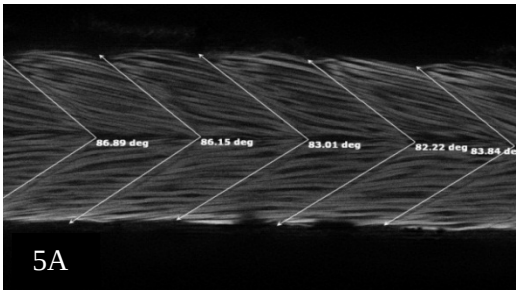


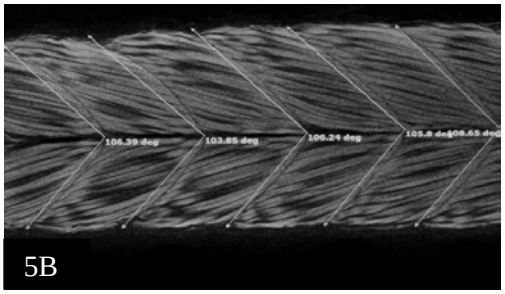
4D

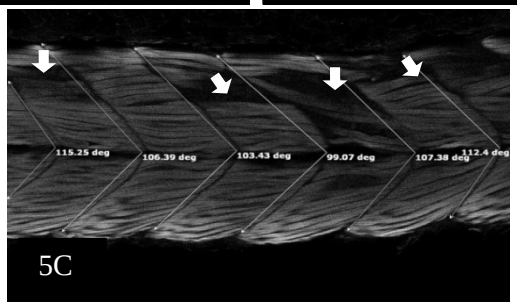
Condition	Boundaries Crossed	Total MTJ Boundaries
Wildtype Control	0	282
nrk2b transgenics	0	201
nrk2b transgenic/*dag1* morphants	0	57

Figure 4. No boundary crossings occur in *nrk2b* transgenic*/dag1* morphants. Zebrafish are imaged, side mounted, and phallodin stained with MTJ angle measurements at 72 hpf. (A) AB wildtype control with no boundary crossings across the MTJs. (B) A *nrk2b* overexpressing transgenic with no boundary crossings across the MTJs. (C) A *nrk2b* transgenic*/dag1* morphant with no boundary crossings across the MTJs. (D) Table indicates neither the *nrk2b* overexpressing transgenic control nor *nrk2b* transgenic*/dag1* morphants display boundary crossing in their phenotypes. These data call attention to the observation boundary crossings are distinct from the wide MTJ angle phenotype.

Figure 5







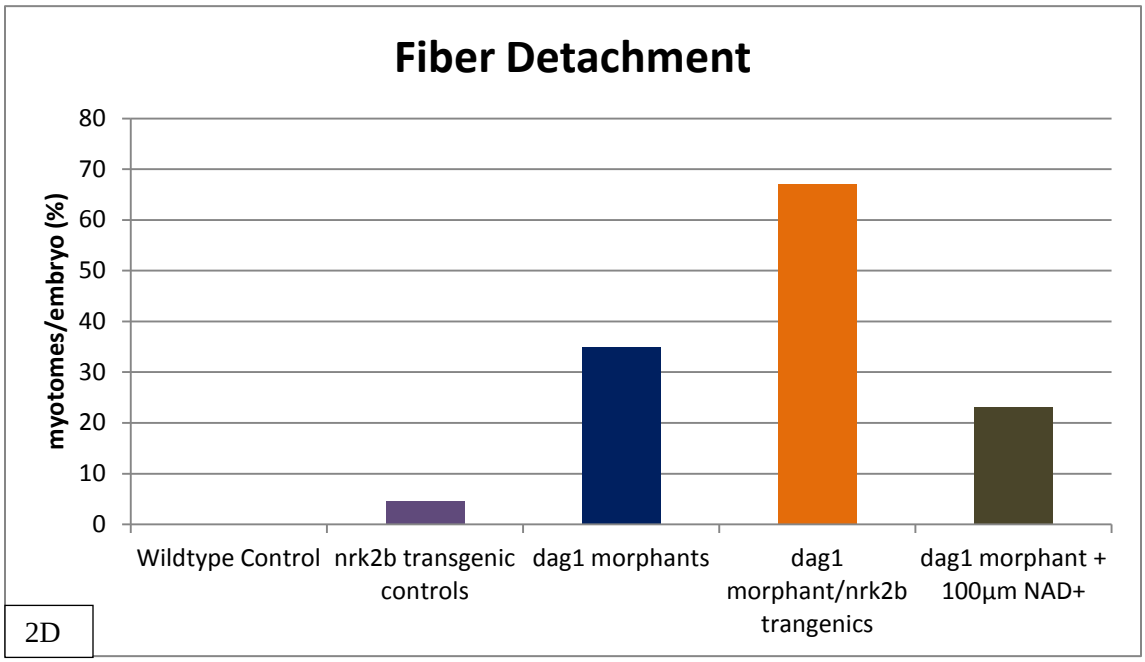


Figure 5. Fiber detachment present in *nrk2b* transgenic/*dag1* morphants. Zebrafish are imaged, side mounted, and phallodin stained with MTJ angle measurements at 72 hpf. (A) AB wildtype control with no fiber detachment. (B) A*nrk2b* transgenic control with no fiber detachment. (C) A*nrk2b* transgenic/*dag1* morphant with detached muscle fibers (indicated by white arrows). (D) Graph demonstrates the *nrk2b* transgenic controls have an outlier percentage of fiber detachment. Compared to *dag1* morphants and *dag1* morphants rescued with NAD+, *nrk2b* transgenic/*dag1* morphants have exacerbated fiber detachment (*dag1* morphantand *dag1* morphant/NAD+ data from Goody et al., 2010).These data suggest *nrk2b* overexpression does not alleviate dystrophy but may exacerbate dystrophy in DAG1-deficient zebrafish.

Figure 6

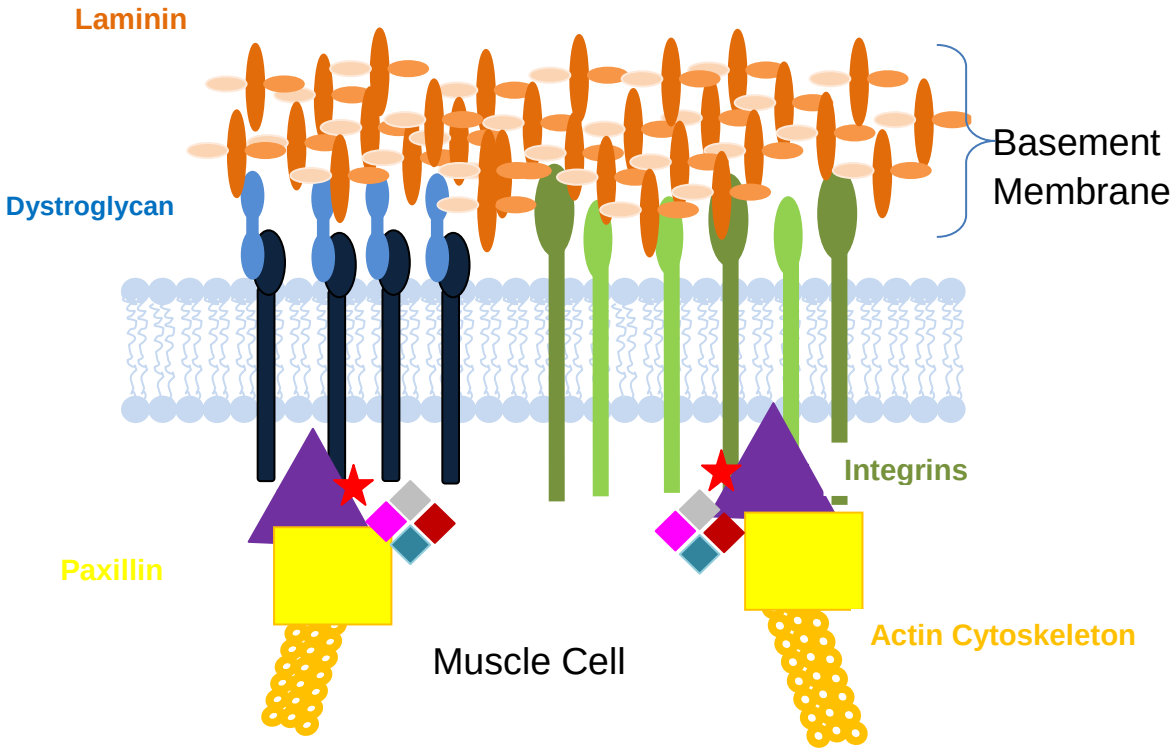


Figure 6.Myotendinous junction components anchoring a muscle cell to the ECM: The basement membrane is substructure of the ECM and one of its constituents is the laminin glycoprotein heterotrimers. Muscle cells' actin cytoskeleton attach to the basement membrane through transmembrane proteins like the laminin receptors dystroglycan and integrins. Intracellular proteins, like paxillin, localizing to the sub-membrane zone organize actin cytoskeleton adhesion.

Figure 7

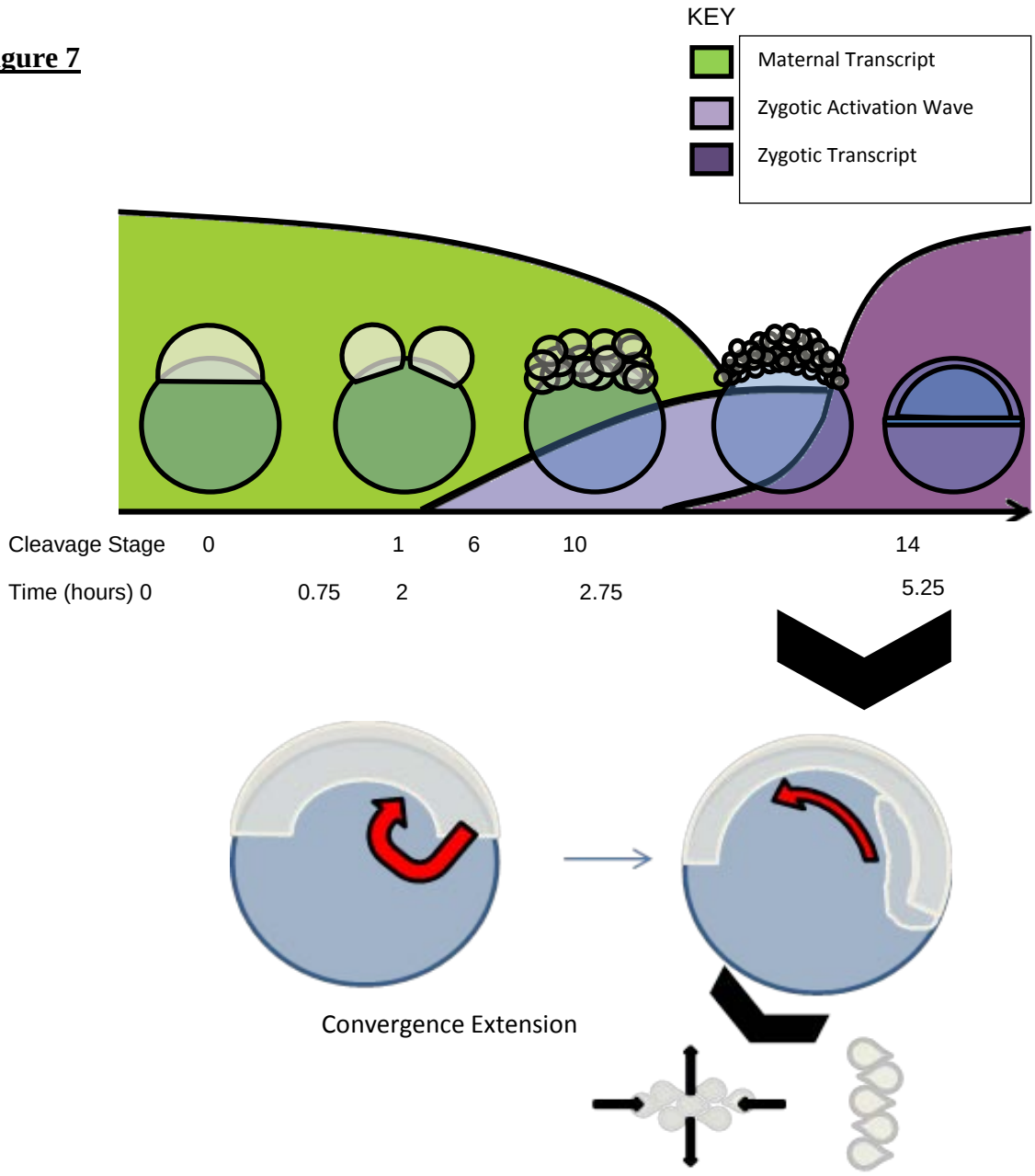


Figure 7.Cell movements occurring in the window of time between zygotic *nrk2b* overexpression and addition of NAD+. In MZT, maternal transcripts (green wave) degrade while zygotic gene expression (purple waves), like *nrk2b* overexpression, takes over at 3 hpf in zebrafish. Cell movements during gastrulation happen after 3 hpf. In convergence extension, the elongated body axis forms as cell migrations narrow medio-lateral and elongate anterior-lateral axes. These movements may be disrupted in and cause the phenotype in *nrk2b* overexpressing transgenics. Referenced: (Tardos and Lipshitz, 2009; Wolpert, 2007).

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Appendix

Animal Care Certification

Completed on1/18/12

About the Author

Anna Burgess was born in July of 1990 and raised in Dedham, Maine. This is not so very far from where she would study double major in literary analysis and biology at the University of Maine at Orono. Her time in the Henry lab was her introduction to thefield of biomedical research. There she learned to love probing the mysteries of biological life. She also had time to work in other labs, live and study in Dunedin, New Zealand, find room for music and tramping, and delve into a growing passion for sustainable agriculture. In the year followinggraduation, she plans to travel and work on farms across West Virginia and Nepal. Beginning in June of 2013, she will start an internship at Four Season Farm in Harborside, Maine for a time of learning, and deciding on graduate school programs.